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(54) Title: SEQUENCES FOR DIFFERENTIAL DIAGNOSTIC OF *EHRLICHIA RUMINANTIUM* AND USE THEREOF

(57) Abstract: The invention provides genes that are unique either to *Ehrlichia ruminantium* strain Gardel or to *Ehrlichia ruminantium* strain Welgevonden, or allelic couples which are present in both strains but whose sequences differ between the two strains, as genetic markers to differentiate between these two strains. The invention also provides diagnostic methods using said genetic markers.

SEQUENCES FOR DIFFERENTIAL DIAGNOSTIC OF *EHRLICHIA RUMINANTIUM* AND USE THEREOF

Rickettsia are intracellular pathogenic bacteria responsible for various diseases on Humans and animals. *Rickettsia* are transmitted by arthropods, most frequently ticks, lice and mites, and cause major illnesses such as epidemic typhus or Rocky Moutain spotted fever. The genus *Ehrlichia* comprises several species pathogenic for humans and mammals such as *E. chaffeensis*, responsible for Human monocytic ehrlichiosis, *E. canis*, the causing agent of canine monocytic ehrlichiosis, or *E. phagocytophillia*, the agent of Human granulocytic ehrlichiosis.

Another species, *Ehrlichia ruminantium*, formerly known as *Cowdria ruminantium*, is the causing agent of heartwater or cowdriosis, an economically important disease of domestic ruminants. Heartwater can cause up to 80 % mortality in susceptible animals. *E. ruminantium* is transmitted by *Amblyomma* ticks and is present in Sub-Saharan Africa and surrounding islands, including Madagascar. Heartwater is also present in several Caribbean islands and is threatening the American mainland.

Serological diagnostic tests of heartwater using crude antigens from whole bacteria detect false positive reactions due to common antigenic determinants. ELISA-based and serological diagnostics have been developed using the Map 1 (WO 9914233; Sumption *et al.* Clin Diagn Lab Immunol. 10: 910-916, 2003) and the GroEL (WO 9914233) antigens. Other peptides for serological diagnostic have been described (US 20020004051, US 20020132789, WO 02/066652). Although they have dramatically improved specificity, they still display cross reaction with *E. canis* and *E. chaffeensis*. Furthermore, the life span of anti-Map 1 antibodies is rather short.

PCR-based diagnostic methods represent methods of choice for the sensitive and specific detection of *Ehrlichia* in clinically reactive or asymptomatic carrier ruminants, as well as in vectors. However, in the field, hosts and vectors can be co-infested by several parasites and the diversity of pathogen species is further complicated by the existence of extensive intra-species diversity. Improved methods are required to discriminate between strains of differing pathogenesis.

Vaccination against heartwater has long been based on ""infection and treatment". Naïve animals are inoculated with blood containing virulent organisms, a procedure which carries a high risk of uncontrolled clinical reactions and the inadvertent spread of undesirable parasites and viruses. A first generation of cowdriosis inactivated vaccine based on cell-cultured derived elementary bodies was developed. Although representing a considerable improvement and the first heartwater vaccine acceptable for widespread use, the level of protection conferred is still not fully satisfactory. Indeed, all animals develop a clinical reaction at challenge despite vaccination. Furthermore, livestock also faces challenge by genetically and antigenically diverse strains.

Diversity of *E. ruminantium* is a key problem which has been recognized for a long time, but insufficient information is available for optimum vaccine formulation and specific diagnostic. The diversity of *E. ruminantium* was demonstrated at the antigenic level by cross-immunisation studies. Variable antigens were identified by ELISA and immunoblot using cross-absorbed immune sera. Genetic diversity was later demonstrated when sequencing the Map 1 gene which showed a high degree of sequence heterogeneity concentrated in three hypervariable regions. This DNA polymorphism was shown to correlate with antigenic polymorphism. Genomic polymorphism was also detected using RAPD and RFLP markers. The map1 gene initially considered as a good marker for geographic diversity, was recently shown not to be geographically constrained. Furthermore, there is no evidence of evolution of *map1* under positive selection pressure. Map1 was therefore reported as not being important for evasion of host immune response.

It is shown herein that between two strains of *Ehrlichia ruminantium*, i.e. strain Gardel and strain Welgevonden, the protection acquired through vaccination with one strain is without effect towards the other one. When an animal is protected by vaccination against the strain Gardel it remains susceptible to lethal infection with the strain Welgevonden, i.e. there is no cross protection between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

Thus, it is important to provide means and diagnostic tools allowing not only to identify *E. ruminantium* but also to differentiate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

The invention provides genetic markers to differentiate between these two strains, and diagnostic methods using said genetic markers. More specifically, the invention identifies large genetic deletions that are specific either to *E. ruminantium* strain Gardel or to *E. ruminantium* strain Welgevonden, and provide means to distinguish between these two strains by detecting the presence or absence of at least one of said deletions. These deletions may result in the loss of a whole gene, or only of a part of it.

According to a first embodiment, the invention provides gene sequences present in only one of the two differing strains of *Ehrlichia ruminantium*. These sequences represent regions of high strain-specificity for development of diagnostic tools. They will be defined herein as "orphan genes", or "unique genes". They correspond to CDS which have no counterpart in the other strain.

According to a second embodiment, the present invention provides couples of genes which are present in both strains but whose sequences differ between the two strains, due to one or several mutations, including in particular deletions that represent a good target for strain-specific detection. These couples of genes will be defined herein as "allelic couples". The longest member of an allelic couple, that appears, on the basis of sequence data, to encode a potentially functional protein will be defined herein as the "native gene", or

the "native allele". The truncated member of the allelic couple, which appears, on the basis of sequence data, to encode a modified protein which is potentially non-functional, or functionally altered , will be defined herein as the "mutant gene", or the "mutant allele".

The CDS corresponding to the native gene in one strain may have, depending on the type of mutation, one or two counterparts in the other strain. More specifically, in case wherein the mutation induces a frameshift where the initial reading frame is changed due to deletion of bases and results in a shift of the frame, a second or additional CDS might be predicted by the annotation software package (i.e. GenoStar package – www.genostar.org) downstream from the site of mutation. This additional CDS is not a novel gene *per se* but merely the continuation of part of the original full length gene which was shortened by the mutation. If the part of the coding sequence located downstream from the mutation meets the prediction requirements regarding minimal size and presence of a start and a stop codon, it will be considered by the annotation software as a "novel" CDS. A specific number will therefore be attached to this additional CDS although it is only part of the initial full length gene and does not correspond to a biologically distinct gene. As a consequence, in some cases, the beginning of the mutant counterpart of the native gene will be found within a first CDS, while the end of the mutant counterpart of the native gene will be found within a second CDS.

The invention provides methods of detecting *Ehrlichia ruminantium* and, advantageously, of discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden, using any of the orphan genes or allelic couples defined above, or any combination thereof.

Accordingly, a first object of the invention is a method for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden, wherein said method comprises the detection of the presence or the absence, in the bacteria to be tested, of at least one orphan gene selected among:

ERGA_CDS_04340 (SEQ ID NO: 1)
ERGA_CDS_04980 (SEQ ID NO: 2)
ERGA_CDS_05590 (SEQ ID NO: 3)
ERGA_CDS_05600 (SEQ ID NO: 4)
ERGA_CDS_07580 (SEQ ID NO: 5)
ERWE_CDS_08330 (SEQ ID NO: 6)

ERGA_CDS_04340, ERGA_CDS_04980, ERGA_CDS_05590, ERGA_CDS_05600, and ERGA_CDS_07580 are found only in the genome of *E. ruminantium* strain Gardel.

ERWE_CDS_08330 is found only in the genome of *E. ruminantium* strain Welgevonden.

The method of the invention may comprise the detection of a single orphan gene among those listed above, or the detection of any subset of 2, 3, 4, 5, or 6 of these genes.

According to a preferred embodiment of the method of the invention, it comprises the detection of at least one gene selected among ERGA_CDS_05590, and ERGA_CDS_07580.

Another method provided by the present invention for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden relies on the detection of a member of an allelic couple of genes, as defined above.

Accordingly, a second object of the invention is a method for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden, wherein said method comprises the detection in the bacteria to be tested, of one of the members of at least one allelic couple of genes selected among:

- a couple consisting of ERGA_CDS_00120 (SEQ ID NO: 7) and ERWE_CDS_00120 (SEQ ID NO: 8);
- a couple consisting of ERGA_CDS_01350 (SEQ ID NO: 9) and ERWE_CDS_01390 (SEQ ID NO: 10);
- a couple consisting of ERGA_CDS_05740 (SEQ ID NO: 11) and ERWE_CDS_05830 (SEQ ID NO: 12);
- a couple consisting of ERGA_CDS_04500 (SEQ ID NO: 13) and ERWE_CDS_04590 (SEQ ID NO: 14) + ERWE_CDS_04600 (SEQ ID NO: 15)
- a couple consisting of ERGA_CDS_05350 (SEQ ID NO: 16) and ERWE_CDS_05460 (SEQ ID NO: 17) + ERWE_CDS_05470 (SEQ ID NO: 18)
- a couple consisting of ERGA_CDS_07330 (SEQ ID NO: 19) and ERWE_CDS_07410 (SEQ ID NO: 20).

ERGA_CDS_00120, ERGA_CDS_01350, ERGA_CDS_05740, ERGA_CDS_04500 and ERGA_CDS_05350, are alleles herein defined as native alleles, that are found in strain Gardel. ERWE_CDS_07410 is an allele herein defined as a native allele, that is found in strain Welgevonden.

The method of the invention may comprise the detection of a member of a single allelic couple among those listed above, or the detection of a member of each allelic couple in a combination of 2, 3, 4, 5, or 6 of those listed above.

In the allelic couples disclosed above, the mutant allele differs from the native allele by the presence of a deletion resulting in the loss of part of the transcribed region corresponding to the central part of the native coding sequence. This deletion generates a truncation, that can be accompanied by a frameshift.

Three main regions can therefore be considered. These regions are presented in Figure 1. The first region, named Zone 1, is the 5' region of the gene up to the beginning of the deletion in the mutant gene. Zone 1 is a conserved region of high similarity between *E.*

ruminantium strain Gardel and *E. ruminantium* strain Welgevonden. An oligonucleotide designed to match this region will recognize both strains. Zone 2, corresponds to the region of deletion in the mutant gene and therefore only the native allele bears a sequence in this region and can be recognized by an oligonucleotide designed to match this region. Zone 3 is the second conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. In this region also, an oligonucleotide designed to match Zone 3 will recognize both strains. In the mutant allele, Zone 1 adjoins Zone 3. An oligonucleotide designed to match the junction, i.e the regions of Zone 1 and Zone 3 immediately flanking the deletion, will recognize only the mutant strain.

Advantageously, the method of the invention comprises the detection, in the bacteria to be tested, of the presence or the absence of at least one orphan gene among those cited above, and the detection of at least one of the members of an allelic couple among those cited above.

Still another object of the invention is a method for detecting *E. ruminantium* wherein said method comprises the detection in the bacteria to be tested, of the presence or the absence of any of the members of at least one allelic couple of genes selected among :

SEQ ID NO: 7 and SEQ ID NO: 8;
SEQ ID NO: 9 and SEQ ID NO: 10;
SEQ ID NO: 11 and SEQ ID NO: 12;
SEQ ID NO: 13 and SEQ ID NO: 14+15;
SEQ ID NO: 16 and SEQ ID NO: 17+18;
SEQ ID NO: 19 and SEQ ID NO: 20.

The invention also provides tools for detecting the presence or the absence of the orphan genes listed above, as well as tools for detecting the allelic couples of genes listed above, and differentiating their members.

These tools include in particular isolated polynucleotides defined by the sequences SEQ ID NO:1 to 20 disclosed above or their complement, as well as fragments of at least 15 consecutive bp, preferably at least 18 consecutive bp, thereof. They also include polynucleotides that hybridize selectively, under stringent hybridization conditions, with one or two of the polynucleotides defined by the sequences SEQ ID NO: 1 to 20 described above, or with the complement thereof, without hybridizing to other sequences within the genome of *Ehrlichia ruminantium.*

A polynucleotide that hybridize selectively with a given target sequence, is herein defined as a polynucleotide which does not hybridize, under the same hybridization conditions, with other sequences within the genome of *Ehrlichia ruminantium.*

Stringent hybridization conditions are defined as conditions that allow hybridization of only highly homologous sequences (i.e sequences having at least 90% and

preferably at least 95 to 100% identity). It is known in the art that nucleic-acid hybridization is affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of mismatched bases between the hybridizing nucleic acids. Generally, stringent conditions for a given sequence can be obtained by performing hybridization at a temperature of about 10 to 20°C lower than the melting point (T_m) for the hybrid formed by said sequence and its exact complement, and at least one wash at a temperature of about 1 to 10°C lower, preferably at a temperature of about 1 to 5°C lower than the T_m for the hybrid formed by said sequence and its exact complement.

The polynucleotides of the invention can be divided in 3 sub-categories:

- polynucleotides specific to one of the orphan genes described above: said polynucleotides are fragments of anyone of the sequences SEQ ID NO: 1 to 6 or of its complement, as well as polynucleotides able to hybridize selectively, under stringent conditions, with anyone of the sequences SEQ ID NO:1 to 6 or with its complement ;

- polynucleotides common to both members of one of the allelic couples defined above: said polynucleotides are fragments, shared by both members of a given allelic couple, of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement, or polynucleotides able to hybridize selectively, under stringent hybridization conditions, with a region shared by both members of a given allelic couple, of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement. These polynucleotides are useful to detect *E. ruminantium*, and may also be used, as illustrated below, in methods for discriminating between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

- polynucleotides specific to one of the members of one of the allelic couples disclosed above: said polynucleotides are fragments of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement, that are present in only one of the members of a given allelic couple, or polynucleotides able to hybridize selectively, under stringent hybridization conditions, with a region of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement, that is present in only one of the members of a given allelic couple. For a given allelic couple, these polynucleotides include those consisting of Zone 2 (or fragments thereof) as well as those spanning the junction between Zone 1 and Zone 3. In the case of a polynucleotide spanning the junction between Zone 1 and Zone 3, it will preferably be chosen in such a way that about half of its sequence is derived from Zone 1 and about half of its sequence is derived from Zone 3.

Polynucleotides of the invention include in particular nucleic acid probes or PCR primers.

For use as PCR primers one will generally chose oligonucleotides of about 18 to 25 bp. A variety of procedures and softwares for designing appropriate primers for a target region are available. Thus, one skilled in the art can easily design, based on the

information provided by the present invention, sets of PCR primers suitable to generate an amplification product specific to anyone of the orphan genes listed above, or to generate amplification products from both members of anyone of the allelic couples listed above, or to generate an amplification product from only one of the members of said allelic couple. By way of non-limitative example of oligonucleotide design software suitable for obtaining PCR primers of the invention, one can mention the software Vector NTI Advance 9.0 (Informax).

For use as nucleic acid probes, one will prefer polynucleotides that comprise at least 30 bp, preferably at least 50 bp, and up to the whole length of the target sequence. Many softwares and procedures are available to the one skilled in the art, who can easily design, based on the information provided by the invention, suitable polynucleotides useful for efficiently discriminate *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden.

Polynucleotides of the invention can be DNA, RNA, or synthetic analogs, such as peptide nucleic acids, wherein the ribose phosphodiester backbone of the polynucleotide is replaced with a pseudo-peptide (polyamide) backbone.

They can be obtained by classic methods, well known to the one skilled in the art, such as chemical synthesis, restriction enzyme digestion, by PCR amplification, etc. They can also be labeled, by radioactive or cold labeling. Numerous protocols for polynucleotide synthesis or labeling are well known in the art (cf. for instance Sambrook and Russell, 2001 : *Molecular Cloning: A Laboratory Manual,* 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

For the purpose of carrying out the invention, the polynucleotides of the invention can be used in many different ways, according to the various techniques for detection of a target nucleic acid sequence based on selective nucleic acid hybridization which are available in the art (cf. for instance Sambrook and Russell, 2001, mentioned above).

The methods of the invention can be performed either on whole bacteria previously lysed, or on nucleic acid (genomic DNA, cDNA or mRNA) isolated from said bacteria.

By way of example, polynucleotides of the invention can be used to detect *E. ruminantium*, and advantageously, to differentiate between *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden, in Southern hybridization, blot hybridization, Northern hybridization, colony hybridization on bacterial colonies, PCR amplification etc. They can be used individually in separate reactions, or they can be combined by 2 or more for use in a same reaction mixture. In this case, they will be labelled in order to be distinguished from each other, and/or they will be spatially separated by immobilization on different spots of a solid phase matrix.

According to a particularly preferred embodiment, polynucleotides of the invention are used as immobilized probes in DNA arrays. For this use, polynucleotides of at least 30 bp, preferably of at least 50 bp will be preferred. Appropriate polynucleotides may be designed from the target sequences provided by the invention, by methods known in themselves, for instance using OligoArray 2.1 (Rouillard et al.. Nucleic Acids Research. 31 : 3057-3062, 2003).

Non-limitative examples of polynucleotides of the invention that can be used as nucleic acid probes for detecting the orphan genes defined above are: oligo-ERGA-4340, oligo-ERGA-4980, oligo-ERGA-5590, oligo-ERGA-5600, oligo-ERGA-7580, and oligo-ERWE-8330, that respectively hybridize selectively with the orphan genes ERGA_CDS_04340, ERGA_CDS_04980, ERGA_CDS_0559O, ERGA_CDS_05600, ERGA_CDS_07580 and ERWE_CDS_08330.

Other non-limitative examples of polynucleotides of the invention that can be used as nucleic acid probes for detecting the same orphan genes are the amplicons PCR-oligo-ERGA-4340, PCR-oligo-ERGA-4980, PCR-oligo-ERGA-5 590, PCR-oligo-ERGA-5600, PCR-oligo-ERGA-7580, and PCR-oligo-ERWE-8330 that respectively hybridize selectively with the orphan genes ERGA_CDS_0434O, ERGA_CDS_04980, ERGA_CDS_05590, ERGA_CDS_05600,. ERGA_CDS_07580 and ERWE_CDS_08330.

The invention also includes nucleic acid probes useful for detecting *E. ruminantium* through the detection of any of the members of one or several allelic couple(s) of genes defined above. These probes can be directed to the Zone 1 region (P-Z-1 probe), or to the Zone 3 region (P-Z-3 probe), of the targeted allelic couple. A combination of a P-Z-1 probe and of a P-Z-3 probe can also be used.

The invention also includes nucleic acid probes useful for differentiating between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden through the discrimination between the members of one or several allelic couple(s) of genes defined above. These probes are specific for the junction between Zone 1 and Zone 3, or preferably, for the Zone 2 region (P-Z-2 probe) of the targeted allelic couple. They are more particularly useful for discrimination between members of allelic couples where the size of the deletion in zone 2 is sufficiently important to allow its easy detection by DNA hybridation.

These allelic couples include:

- the couple consisting of ERGA_CDS_01350 and ERWE_CDS_01390;
- the couple consisting of ERGA_CDS_04500 and ERWE_CDS_04590+ ERWE_CDS_04600;
- the couple consisting of ERGA_CDS_05350 and ERWE_CDS_05460+ ERWE_CDS_05470.

According to a preferred embodiment, the invention includes triplets of nucleic acid probes useful for discrimination between members of allelic couples, comprising

a probe specific of the Zone 1 region (P-Z-1 probe), a probe specific of the Zone 2 region (P-Z-2 probe), and a probe specific of the Zone 3 region (P-Z-3 probe).

Non limitative examples of said triplets of probes, designated MutERWE multiplexes, comprise the following oligonucleotides:

- MutERWE-1390N1, MutERWE-1390N2, and MutERWE-1390N3;
- MutERWE-4590N1, MutERWE-4590N2, and MutERWE-4600N3;
- MutERWE-5460N1, MutERWE-5460N2, and MutERWE-5460N3.

Advantageously, the nucleic acid probes of the invention are used in DNA arrays allowing to test simultaneously several genes of *E. ruminantium*, wherein said genes include at least one of the orphan genes defined above, and/or at least one member of any of the allelic couples defined above.

The invention also encompasses said DNA arrays.

DNA arrays of the invention are characterized in that they comprise at least one polynucleotide of the invention of at least 30 bp, preferably at least 50 bp, selected among :

- a polynucleotide specific to anyone of the orphan genes defined above;
- a polynucleotide common to both members of anyone of the allelic couples defined above (i.e targeted to the Zone 1 or the Zone 3 region of said allelic couple);
- a polynucleotide specific to one of the members of anyone of the allelic couples disclosed above (i.e targeted either to the Zone 2 region or to the junction between Zone 1 and Zone 3 of said allelic couple).

Advantageously, DNA arrays of the invention comprise a combination of 2 or more of said polynucleotides.

Thus, DNA arrays of the invention allow to test in an *E. ruminantium* strain to be analyzed, various combinations of genes that can include from one to all of the orphan genes and/or from one to all of allelic couples defined above.

Non-limitative examples of DNA arrays of the invention are:

i) a DNA array comprising at least one polynucleotide selected among the following: PCR-oligo-ERGA-4340, PCR-oligo-ERGA-4980, PCR-oligo-ERGA-5590, PCR-oligo-ERGA-5600, PCR-oligo-ERGA-7580, and PCR-oligo-ERWE-8330, or any combination of 2, 3, 4, 5, or 6 of these polynucleotides;

ii) a DNA array comprising at least one polynucleotide selected among the following: oligo-ERGA-4340, oligo-ERGA-4980, oligo-ERGA-5590, oligo-ERGA-5600, oligo-ERGA-7580, and oligo-ERWE-8330, or any combination of 2, 3, 4, 5, or 6 of these polynucleotides.

iii) a DNA array comprising at least one polynucleotide selected among the following: MutERWE-1390N2, MutERWE-4590N2, and MutERWE-5460N2;

iv) a DNA array comprising at least one polynucleotide selected among the following: MutERWE-1390N1, MutERWE-1390N3, MutERWE-4590N1, MutERWE-4600N3, MutERWE-5460N1 and MutERWE-5460N3;

v) a DNA array comprising any combination of at least one of the polynucleotides listed in iii) with at least one of the polynucleotides listed in iv);

vi) a DNA array comprising any combination of at least one of the polynucleotides listed in i) or ii) with at least one of the polynucleotides listed in iii) and/or at least one of the polynucleotides listed in iv);

A large range of methods, protocols and techniques exist to develop and probe DNA arrays and read and analyse data. The person expert in the art has easy access to a large literature on the DNA array technology and can easily implement any kind of DNA array approach to identify *E. ruminantium*, and/or discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden using information provided in the invention. One can find further information and start a broader literature review if needed from the following references: Lipshutz *et al.*, Nat Genet. 21: 20-24, 1999 ; Kiechle and Holland-Staley, Arch. Pathol. Lab. Med. 127: 1089-1097, 2003 ; Rast *et al.*, Dev Biol. 228: 270-286, 2000 ; Manduchi *et al.*, Bioinformatics 16: 685-698, 2000 ; Mäder *et al.*, J. Bacteriol. 184: 4288-4295, 2002 ; Bowtell, Nat. Genet. 21: 25-32, 1999 ; WO 2004/061111.

The present invention also includes combinations of polynucleotides of the invention that can be used as sets of PCR primers.

Non limitative examples of sets of primers that can be used to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden by detecting the presence or the absence of the orphan genes described above are: P-ERGA-4340-A/P-ERGA-4340-B; P-ERGA-4980-A/P-ERGA-4980-B; P-ERGA-5590-A/P-ERGA-5590-B; P-ERGA-5600-A/P-ERGA-5600-B; P-ERGA-7580-A/P-ERGA-7580-B; P-ERWE-8330-A/P-ERWE-8330-B.

Other examples of sets of primers of the invention are those which allow depending on the way they are used, either to detect *E. ruminantium,* or to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

This type of sets of primers includes combinations of polynucleotides common to both members of anyone of the allelic couples defined above under sequences SEQ ID NO: 7 to 20. Typically, said combinations comprise:

- at least one polynucleotide specific of the region upstream from the mutation (i.e Zone 1); and/or

- at least one polynucleotide specific of the region downstream from the mutation (i.e Zone 3).

These sets of PCR primers allow obtaining an amplification product from both members of the allelic couple. In this case, the discrimination between the native and the mutant allele will be performed on the basis of the difference of size and/or of sequence between the amplification products, by way of example through their RFLP patterns after appropriate enzymatic digestion.

Non-limitative examples of such sets of primers are P-WEGA-120-S/P-WEGA-120-AS; P-WEGA-1350-S/P-WEGA-1350-AS; P-WEGA-4500-S/P-WEGA-4500-AS; P-WEGA-5350-S/P-WEGA-5350-AS; P-WEGA-5740-S/P-WEGA-5740-AS; P-WEGA-7410-S/P-WEGA-7410-AS.

Based on the information provided in the invention, a person skilled in the art can easily design other primers, detect other sequences and select different sets of restriction enzymes. A large range of RFLP strategies and techniques exist, some of them being for instance associated to PCR, to labeled probes, to Southern-blot analysis or to DNA sequencing, and a person expert in the art can easily implement a whole range of approaches to detect *E. ruminantium* or to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden using information from the invention. Similarly, a broad range of techniques exist to obtain, separate and analyze restriction fragments for RFLP analysis. Data provided in example 9 are compatible with any kind of technique for separation and analysis which are well described in the literature and known to a person expert in the art.

Another type of sets of primers of the invention are those which allow to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden, by detecting the presence or the absence of an amplification product.

This type of sets of primers includes polynucleotides common to both members of anyone of the allelic couples defined above under sequences SEQ ID NO: 7 to 20, and polynucleotides specific to one of the members of said allelic couple.

Typically, a combination of this second type comprises:

- at least one polynucleotide specific to the mutated region (i.e Zone 2, or the junction between Zone 1 and Zone 3), and thus specific to one of the members of the allelic couple ; and
- at least one polynucleotide common to both members of the allelic couple, and specific of the region upstream from the mutation (i.e the Zone 1); and/or
- at least one polynucleotide common to both members of the allelic couple, and specific of the region downstream from the mutation (i.e the Zone 3).

Examples of such combinations include for instance:

- pairs of primers, where the sense primer recognizes the Zone 1 region (P-Z-1 primer) and the antisense primer recognises the Zone 2 region (P-Z-2-AS primer), or the sense primer recognises the Zone 2 region (P-Z-2-S primer) and the antisense primer recognises the Zone 3 region (P-Z-3-AS primer).

- pairs of primers where the sense primer recognizes the Zone 1 region (P-Z-1 primer) and the antisense primer recognises the junction between Zone 1 and Zone 3 (P-Z-1/3-AS primer), or the sense primer recognises the junction between Zone 1 and Zone 3 (P-Z-1/3-S primer) and the antisense primer recognises the Zone 3 region (P-Z-3-AS primer).

Such combinations are useful for instance to discriminate *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden, by carrying out a simple PCR in parallel on both strains, on the basis of the absence or presence of a PCR product.

- triplets of primers, wherein a sense primer recognizes the Zone 1 region (P-Z-1 primer), a first antisense primer recognises the Zone 2 region (P-Z-2-AS primer), or junction between Zone 1 and Zone 3 (P-Z-1/3-AS primer) and a second antisense primer recognises the Zone 3 region (P-Z-3 region). Such a combination allows discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden by multiplex PCR performed in parallel in the 2 strains.

Using such combinations of three primers in the same PCR reaction yields differential patterns for *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

Non-limitative examples of such pairs or triplets of primers, and of their use for discriminating *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden by simple PCR and multiplex PCR are:

- P-Z-1-ERGA-120; P-Z-2-ERGA-120-S; P-Z-2-ERGA-120-AS; P-Z-3-ERGA-120;
- P-Z-1-ERGA-1350; P-Z-2-ERGA-1350-S;P-Z-2-ERGA-1350-AS;P-Z-3-ERGA-1350;
- P-Z-1-ERGA-4500;P-Z-2-ERGA-4500-S;P-Z-2-ERGA-4500-AS;P-Z-3-ERGA-4500;
- P-Z-1-ERGA-5350; P-Z-2-ERGA-5350-S;P-Z-2-ERGA-5350-AS;P-Z-3-ERGA-5350;
- P-Z-1-ERGA-5740;P-Z-2-ERGA-5740-S;P-Z-2-ERGA-5740-AS;P-Z-3-ERGA-5740;
- P-Z-1-ERWE-7410;P-Z-2-ERWE-7410-S;P-Z-2-ERWE-7410-ASP-Z-3-ERWE-7410.

For all these primers, an amplification product is obtained only from the native member of the allelic couple when a P-Z-2-S primer or a P-Z-2-AS primer is used.

- P-Z-1-ERGA-4500;P-Z-1/3-ERGA-4500-S; P-Z-1/3-ERGA-4500-AS;P-Z-3-ERGA-4500;

For these primers, an amplification product is obtained only from the mutant member of the allelic couple when a P-Z-1/3-S primer or a P-Z-1/3-AS primer is used.

These combinations are meant to exemplify the approach and do not limit the invention. A person skilled in the art can easily define other primers combinations and/or other primer orientations which will lead to the detection of *E. ruminantium* or to a clear discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

The invention also comprises diagnostic kits for detecting *E. ruminantium* or for discriminating between strain Gardel and strain Welgevonden of *E. ruminantium*,

wherein said kits comprise at least a nucleic acid probe and/or at least a set of primers of the invention.

The polynucleotides of the invention are also useful to produce polypeptides specific of either *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. Expression of foreign genes in prokaryotic or eukaryotic systems for production of proteins is well known to the expert in the art. A broad range of techniques exist in the literature and/or are available from commercial companies to express foreign genes and produce and purify proteins.

The invention also includes the polypeptides encoded by the sequences SEQ ID NO: 1-20, an in particular the polypeptides encoded by the sequences SEQ ID NO: 1-6, SEQ ID NO: 7, 9, 11, 13, 16, and 20.

The invention also encompasses tools for producing said polypeptides, and in particular :

- a recombinant expression vector comprising a polynucleotide of the invention, selected among the polynucleotides defined by the sequences SEQ ID NO: 1-20, and in particular the polynucleotides defined by the sequences SEQ ID NO: 1-6, SEQ ID NO: 7, 9, 11, 13, 16, and 20;

-an host cell transformed by said expression vector.

The invention also provides, as tools for detecting the orphan genes or the allelic couples of genes listed above, antibodies raised against polypeptides of the invention. Production of polyclonal and monoclonal antibodies is well known to the expert in the art and custom development of antibodies is also provided by companies.

The invention also comprises diagnostic kits for detecting *E. ruminantium* or for discriminating between strain Gardel and strain Welgevonden of *E. ruminantium*, wherein said kits comprise at least a nucleic acid probe and/or at least a set of primers of the invention, or at least an antibody of the invention.

The methods and tools provided by the invention are suitable for use at various stages of the life cycle of *E. ruminantium*, more specifically but not limited to the domestic-ruminants infectious stage, vector-interaction stage or reservoir animals-interaction stage. Preferred utilisations of the methods and tools of the invention include but are not limited to, the detection of *Ehrlichia ruminantium* in a given territory, the strain specific identification of *Ehrlichia ruminantium* in a given territory, the discrimination between strains of *Ehrlichia ruminantium* in a given territory or between different geographical regions, the analysis of strain movements within a region or between geographically distinct regions, the differential presence of strains of *Ehrlichia ruminantium* according to vector species and/or populations or the early detection and risk assessment in regions where potential vectors are present but where the disease has not been recorded yet.

For a better discrimination between *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden, one can advantageously combine the detection of at least one orphan gene or of any combination of orphan genes among those listed herein, with the detection of at least one allelic couple or any combination of allelic couples among those listed herein.

The orphan genes and allelic couples disclosed in the invention are also candidates of choice for the further development of vaccines. Orphan genes and mutant genes are the only genes differing between the strains Gardel and Welgevonden of *E. ruminantium*. Since these two strains do not generate cross protection through vaccination, protective proteins involved must be different in each strain. For instance a protective protein yielding protection against *E. ruminantium* strain Gardel is most likely to be absent or altered in *E. ruminantium* strain Welgevonden, since an animal vaccinated and protected against *E. ruminantium* strain Gardel is not protected and dies when infected with *E. ruminantium* strain Welgevonden. The most obvious group of absent or altered proteins between both strains are those encoded by the orphan or mutant genes. Accordingly, vaccines comprising the polypeptides of the invention proteins will not only protect against heartwater, or cowdriosis, and *E. ruminantium*, but will permit to efficiently protect against non cross-protective strains and prevent the risk of deadly cross-infections.

Specifically examplified herein is the identification of orphan genes and allelic couples from either or both the strains Gardel and Welgevonden from *Ehrlichia ruminantium*, and the use of PCR primers and nucleic acid probes derived from these genes for the development of DNA arrays, as well as the use of PCR primers derived from these genes for single-pair and multiplex PCR. The use of these primers and probes for differentiating *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden is also examplified herein.

The genes and CDS described in these examples are designated according to the annotation of the genome sequences of strains Gardel and Welgevonden, which was performed using the GenoAnnot tool of the GenoStar package (www.genostar.org).

EXAMPLE 1. LACK OF VACCINAL CROSS-PROTECTION BETWEEN *E. RUMINANTIUM* STRAIN GARDEL AND *E. RUMINANTIUM* STRAIN WELGEVONDEN.

The strain Gardel of *E. ruminantium* was isolated in Guadeloupe island in 1982 from a goat injected with an homogenate of a female individual of *A. variegatum* collected on cows (Uilenberg *et al.*, Rev. Elev. Méd. Vét. Pays Trop. 34 : 34-42, 1985). The strain Welgevonden of *E. ruminantium* was isolated in South Africa in 1985 from mice injected with individually homogenised infected field-collected *A. hebraeum* ticks (Du Plessis, 1985). The strain Welgevonden was multiplied in mice for 8 passages (Du Plessis, 1985). *E. ruminantium* was multiplied on bovine umbilical endothelial cell (BUEC) grown in

Glasgow-MEM medium complemented with fœtal calf serum, tryptose-phosphate broth and antibiotics (Bezuidenhout *et al.*, J. Vet. Res. 52: 113-120, 1985) at 37°C, 5% CO2 with a weekly reinfection (Martinez *et al.*, Vet. Parasitol. 67: 175-184, 1990). Unlike the Gardel strain, the strain Welgevonden is highly infective to both rodents (mice) and ruminants through intravenous injection.

Vaccination assays were conducted on Creole goats originating from Les Saintes islands, a heartwater-free region of the Caribbean. Pre-bleed sera of all the animals were negative for anti-*Ehrlichia ruminantium* antibodies as determined by an indirect map-1b ELISA (van Vliet *et al.*, J. Clin. Microbiol. 33: 2405-2410, 1995). After attenuated and virulent challenges, rectal temperature of each animal was daily monitored. *E. ruminantium* strain Gardel was attenuated after more than one hundred successive passages on goat endothelial cells. Virulent Gardel preparation (passage 34 and 42) derived from *in vitro* culture. The supernatant was collected when 70-80% of the cells were lysed by the bacteria for injection to goats. For immunization by infection with ticks and antibiotic treatment, *Amblyomma variegatum* larvae were fed on Gardel *E. ruminantium* infected animal. After moulting, infected nymphs, were engorged on a naive goat which was treated on the third day following hyperthermia with oxytetracycline at a dose of 20 mg/kg of body weight. For immunization with sublethal doses, *in vitro E. ruminantium* strain Gardel preparations (passage 14) were titrated by tissue culture lethal dose 50 (TCLD50) as described previously (Martinez et al., Vet Parasitol. 67: 175-184, 1996). Two-fold serial dilutions of inoculum were prepared to obtain sublethal *E. ruminantium* doses from 10 to 0.625 TCLD50. 5 groups of 5 goats were inoculated i.v. with these doses. Goats which survived following hyperthermia without antibiotic treatment were selected for the experiment.

Goats were vaccinated with *E. ruminantium* strain Gardel using different kinds of vaccines. The first kind of vaccine tested is an attenuated vaccine. In this case, the aggressiveness of the strain was reduced by successive passages on cell culture. In this case, strains are no longer aggressive after 200 serial passages. Strains having undergone less than 100 passages are virulent strains. Attenuated vaccine relies on a strain which is still alive. Another kind of vaccine, inactivated vaccine, was assessed. In this case, the bacteria were killed with sodium azide. Another means of vaccination investigated is infection with pathogenic strains followed by treatments with antibiotics. Animals were infected either with a virulent culture supernatant or by contact with infected ticks. When hyperthermia (fever) appears, the animals are then treated with tetracycline. Another way of vaccinating the animals was to inject sublethal doses of a virulent population and wait for the animal to recover without treatment with antibiotics. Experiments were conducted with animals vaccinated with all the various means of vaccination described above for further homologous or heterologous challenge with a virulent strain.

Vaccination and homologous and heterologous challenge experiments are summarized in Table 1 below.

TABLE 1

Goat NO	Vaccination E. ruminantium strain Gardel Vaccine type	Hyperthermia	Homologous challenge E. ruminantium strain Gardel Hyperthermia	Heterologous challenge E. ruminantium strain Welgevonden Hyperthermia	Death
9412	Attenuated	No	No	Yes (12 D.A.I)	Yes (15 D.A.I)
9642	Attenuated	No	No	Yes (13 D.A.I.)	Yes (15 D.A.I)
9627	Sublethal dose	Yes	No	Yes (13 D.A.I	Yes (15 D.A.I)
9506	Infected ticks AB	Yes 10 D.A.I.	No	Yes (12 D.A.I.)	Yes (15 D.A.I)
0037	Control Yes 5 D.A.I. (A.B. to avoid death)			-	-
0038	Control Yes 6 D.A.I. (A.B. to avoid death)			-	-
9707	Control			Yes (12 D.A.I)	Yes (15 D.A.I)

AB : Treatment with antibiotics
D.A.I : Day After Infection

Experiments were conducted as follows:

Vaccination with attenuated vaccine (Goat NO 9642)

4/11/96 Injection of *E. ruminantium* strain Gardel attenuated after 224 passages.

No clinical reaction was observed indicating that the strain injected was not virulent.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) – 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) – 800 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 12 days post infection and the animal was treated with antibiotic after 15 days to avoid death. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

Vaccination with attenuated vaccine and serial challenge (Goat NO 9412)

28/02/94 Injection of *E. ruminantium* strain Gardel attenuated after 136 passages.

No clinical reaction was observed indicating that the strain injected was not virulent.

7/06/94 Homologous challenge with an a virulent population of *E. ruminantium* strain Gardel (42 passages) – 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) – 2 ml of culture supernatant were injected intravenously

No clinical reaction was observed, indicating that the animal remained protected against *E. ruminantium* strain Gardel after 3 years.

28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) – 800 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 12 days post infection. The animal was not treated with antibiotic and death occurred 15 days after infection with *E. ruminantium* strain Welgevonden. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

Vaccination with sublethal doses (Goat NO 9642)

4/10/96 Infection with a sublethal dose of *E. ruminantium* strain Gardel (0.625 TCLD50 of *E. ruminantium* strain Gardel after 14 passages).

Hyperthermia was observed but the animal survived and recovered without treatment with antibiotics.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) – 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) – 800 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 13 days post infection and the animal was treated with antibiotic after 15 days to avoid death. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

Vaccination with infected ticks (Goat NO 9506)

20/06/96 Infection with ticks infected with *E. ruminantium* strain Gardel.

Hyperthermia was observed 10 days post infection and the animal was treated with antibiotics to avoid death. The animal recovered and survived.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) – 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) – 800 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 12 days post infection and the animal was treated with antibiotic after 15 days to avoid death. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

Control for susceptibility to *E. ruminantium* strain Gardel (Goats NO 0037 and 038)

28/04.98 Infection of naïve goats (not vaccinated) with a virulent population of *E. ruminantium* strain Gardel (32 passages) – 500 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 5 and 6 days post infection. Animals were treated with antibiotic to avoid death. This indicates that the animals were susceptible to *E. ruminantium* strain Gardel.

Control for susceptibility to *E. ruminantium* strain Welgevonden (Goat NO 9707)

28/04.98 Infection of a naïve goat (not vaccinated) with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) – 800 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 12 days post infection. The animal was not treated with antibiotic and death occurred 15 days after infection. This indicates that the animals were susceptible to *E. ruminantium* strain Welgevonden.

EXAMPLE 2. GENERAL FEATURES AND SEQUENCE REFERENCE

For each strain, purified DNA was broken by sonication to generate fragments of differing sizes. After filling up the ends with Klenow polymerase, DNA fragments ranging from 0.5 kb to 4 kb were separated in a 0.8% agarose gel and collected after gelase (Epicentre) digestion of a cut agarose band. Blunt-end DNA fragments were inserted into pBluescript II KS (Stratagene) digested with EcoRV and dephosphorylated. Ligation was performed with the Fast-Link DNA Ligation kit (Epicentre) and competent DH10B *E. coli* were transformed prior to colony isolation on LB-agar+ Ampicillin + Xgal +IPTG. About 15000 clones were isolated for each strain of *E. ruminantium*. Plasmidic DNA from recombinant *E. coli* strains was extracted according to the alkaline lysis method and inserts were sequenced on both strands using universal forward and reverse M13 primers and the ET DYEnamic terminator kit (Amersham). Sequences were obtained with ABI 373 et ABI 377 automated sequencers (Applied Biosystems). Data were analysed and contigs were assembled using Phred-Phrap et Consed software packages (http://www.genome.washington.edu). Gaps were filled in through primer-directed sequencing using custom made primers. A total of about 20000 raw sequence runs were generated and analysed for each *E. ruminantium* strain to generate a full length consensus sequence with a coverage of 6x to 7x.

The genome of *E. ruminantium* strains Gardel and Welgevonden is arranged as a circular chromosome of 1499920 bp and 1512977 bp, respectively. The respective G+C contents for the strains Gardel and Welgevonden is 27.51 % and 27.48 %. The genome of *E. ruminantium* strain Gardel comprises 948 coding sequences of an average size of 1018 bp which represent a total coding surface of 63 % of the whole genome. The genome of *E. ruminantium* strain Welgevonden bears 957 genes of the same average size of 1018 bp. The

genome surface of this strain devoted to coding sequences is 62 %. Both genomes comprise 36 transfer RNAs (tRNA) and 3 ribosomal RNAs (rRNA).

EXAMPLE 3. IDENTIFICATION OF ORPHAN GENES IN THE GARDEL AND WELGEVONDEN STRAINS OF *E. RUMINANTIUM*

The differential analysis of the whole genomes of *E. ruminantium* strains Gardel and Welgevonden showed the presence of coding sequences which are present in only one of the strains and not in the other (orphan gene sequences). Some of the CDS which are unique to *E. ruminantium* strain Gardel and found only in the genome of this strain are presented in Table 2 (Seq ID NO 1 to Seq ID NO 5). One of the CDS which is unique to *E. ruminantium* strain Welgevonden and found only in the genome of this strain is also presented in Table 2 (Seq ID NO 6). Since these sequences are unique to one or the other strain, they clearly represent targets for the differential detection of *E. ruminantium* strain Gardel versus *E. ruminantium* strain Welgevonden.

TABLE 2

CDS name	SeqID	Size (bp)	Annotated function	Strain
ERGA_CDS_04340	Seq ID NO 1	186	unknown	Gardel
ERGA_CDS_04980	Seq ID NO 2	270	unknown	Gardel
ERGA_CDS_05590	Seq ID NO 3	630	unknown	Gardel
ERGA_CDS_05600	Seq ID NO 4	828	unknown	Gardel
ERGA_CDS_07580	Seq ID NO 5	303	unknown	Gardel
ERWE_CDS_08330	Seq ID NO 6	225	unknown	Welgevonden

EXAMPLE 4. IDENTIFICATION OF MUTANT ALLELES IN THE GARDEL AND WELGEVONDEN STRAINS OF *E. RUMINANTIUM*

The differential analysis of the whole genomes of *E. ruminantium* strains Gardel and Welgevonden also showed the presence of coding sequences which are affected by one or several mutations in one of the two strains and for which a non-mutated, functionally active and normal allele is present in the genome of the other strain. These allelic couples of coding sequences are presented in Table 3.

TABLE 3

Mutant allele in	SEQ ID NO	Size (bp)	Nature of mutation	Native allele in:	SEQ ID NO	Size (bp)	Annotated Function
Gardel				Welgevonden			
ERGA_CDS_07330	19	3522	Deletion	ERWE_CDS_07410	20	4122	Unknown
Welgevonden				Gardel			
ERWE_CDS_00120	8	1176	Deletion	ERGA_CDS_00120	7	1266	Unknown
ERWE_CDS_01390	10	2856	Deletion	ERGA_CDS_01350	9	3252	Unknown
ERWE_CDS_05830	12	1659	Deletion	ERGA_CDS_05740	11	1836	Unknown
ERWE_CDS_04590 + ERWE_CDS_04600	14 + 15	873 + 1740	Deletion + Frameshift	ERGA_CDS_04500	13	3570	Unknown
ERWE_CDS_05460 + ERWE_CDS_05470	17 + 18	2361 + 4473	Deletion + Frameshift	ERGA_CDS_05350	16	6903	Unknown

EXAMPLE 5. DIFFERENTIAL DNA ARRAY DETECTION OF STRAINS OF *E. RUMINANTIUM* BASED ON RECOGNITION OF ORPHAN GENES WITH AMPLICONS

PCR primers were designed using Vector NTI Advance 9.0 (Informax).

These oligonucleotides are used to produce amplicons by PCR using *E. ruminantium* strain Welgevonden or strain Gardel DNA as template. Oligonucleotides used in this example as PCR primers, and the resulting amplicons are listed in Table 4. The sequence of the PCR primers is indicated in Table 15A.

TABLE 4

Primer name	Orientation	Position relative to CDS	Size (mer)	CDS	Amplicon (size)
Strain Gardel					
P-ERGA-4340-A	Sense	1-21	21	ERGA_CDS_04340	PCR oligo ERGA 4340 (173 bp)
P-ERGA-4340-B	Antisense	153-173	21	ERGA_CDS_04340	PCR oligo ERGA 4340 (173 bp)
P-ERGA-4980-A	Sense	1-25	25	ERGA_CDS_4980	PCR oligo ERGA 4980 (218 bp)
P-ERGA-4980-B	Antisense	196-218	23	ERGA_CDS_4980	PCR oligo ERGA 4980 (218 bp)
P-ERGA-5590-A	Sense	1-20	20	ERGA_CDS_05590	PCR oligo ERGA 5590 (509 bp)
P-ERGA-5590-B	Antisense	490-509	20	ERGA_CDS_05590	PCR oligo ERGA 5590 (509 bp)
P-ERGA-5600-A	Sense	56-74	19	ERGA_CDS_05600	PCR oligo ERGA 5600 (643 bp)
P-ERGA-5600-B	Antisense	677-698	22	ERGA_CDS_05600	PCR oligo ERGA 5600 (643 bp)
P-ERGA-7580-A	Sense	1-23	23	ERGA_CDS_07580	PCR oligo ERGA 7580 (239 bp)
P-ERGA-7580-B	Antisense	221-239	19	ERGA_CDS_07580	PCR oligo ERGA 7580 (239 bp)
Strain Welgevonden					
P-ERWE-8330-A	Sense	14-38	25	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)
P-ERWE-8330-B	Antisense	173-193	21	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)

Preparation of the amplicons

DNA is extracted from elementary bodies of *E. ruminantium* as described by Perez *et al.* (FEMS Microbiol. Lett. 154: 73-79, 1997). *E. ruminantium* strains are grown in BUEC cells as described in Example 1 above. Elementary bodies are purified from the culture supernatant by differential centrifugation and resuspended in 350 µl of PBS to which is added 150 µl of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM MgCl2 and 125 µg of DNase in order to remove contaminating host cell DNA. After incubation for 90 min. at 37°C, the reaction is stopped by addition of 25 mM EDTA. Elementary bodies are washed three times in water and lysed by overnight incubation at 55°C in a solution of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA, 1.5% SDS and 250 µg/ml of proteinase K. Bacterial DNA is extracted with phenol-choloroform, precipitated with cold ethanol and resuspended in

sterile distilled water. Contamination with cell DNA is evaluated by slot blot hybridization using labelled bovine DNA as a probe and dilutions of bovine DNA (12.5 ng and 25 ng) as positive controls.

Amplicons are amplified from DNA extracted from *E. ruminantium* elementary bodies using the primers described in Table 4. A standard procedure is used to obtain the amplicons through PCR (Sambrook & Russel, "Molecular Cloning :a laboratory manual", 3rd Edition, vol. 2, Chapter 8). PCR amplification of amplicons are obtained by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense and antisense, primer and 3 mM $MgCl_2$ in a final volume of 50 µl. Amplification is carried out under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products, i.e. amplicons, are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

Table 5 below indicates the size and position of the amplicons relative to the corresponding CDS.

TABLE 5

Amplicon			CDS	
Name	Size	Position relative to CDS	Name	Size
Strain Gardel				
PCR-oligo-ERGA-4340	173 bp	1-173	ERGA_CDS_04340	186 bp
PCR-oligo-ERGA-4980	218 bp	1-218	ERGA_CDS_04980	270 bp
PCR-oligo-ERGA-5590	509 bp	1-509	ERGA_CDS_05590	630 bp
PCR-oligo-ERGA-5600	643 bp	56-698	ERGA_CDS_05600	828 bp
PCR-oligo-ERGA-7580	239 bp	1-239	ERGA_CDS_07580	303 bp
Strain Welgevonden				
PCR-oligo-ERWE-8330	180 bp	14-193	ERWE_CDS_08330	225 bp

Preparation of DNA microarrays

Amplicons are spotted using the Amersham Biosciences Lucidea Array spotter on aminosilane-coated mirror glass slides (7 Star, Amersham Biosciences) following the procedure recommended by the supplier. Negative and positive control DNAs are also printed into 24 different sectors of each slide. After printing, the slides are stored at room temperature in a dessicator. Prior to hybridization, DNA is cross-linked to the slides by UV irradiation, washed twice with 0.2% SDS solution and rinced twice with distilled water.

Preparation of labelled DNA from *E. ruminantium*

DNA is extracted from elementary bodies of *E. ruminantium* strain Gardel or strain Welgevonden as described above in this example. Purified DNA is fragmented by sonication as follows. A 500-µl DNA solution in TE buffer (10 mM Tris-HCl pH8.0, 0.3 mM EDTA) is sonicated for 3 cycles of 1 min each at amplitude 5. Samples are placed on ice while sonicated. Samples are incubated on ice for 1 min and centrifuged briefly to

concentration the solution on the bottom of the tube after each cycle of sonication. The size of DNA fragments is checked by 1% agarose-gel electrophoresis under standard conditions. Preferred samples are ranging between 0.3 and 0.5 kb in size. Sonicated DNA from *E. ruminantium* strains Gardel and Welgevonden are labelled with different dyes. DNA from *E. ruminantium* Gardel is labelled with Cy3-dCTP (Amersham Biosciences) whereas *E. ruminantium* Welgevonden is labelled with Cy5-dCTP (Amersham Biosciences). In both cases DNA is labelled by random priming (BioPrime Array CGH Genomic labeling System from Invitrogen) following the procedure recommended by the supplier. 1 µg of sonicated DNA in 21 µl of sterile distilled water is mixed with 20 µl of the random primers solution and incubated at 95°C for 5 min and immediately cooled on ice. Still on ice, the following reagents are added: 5 µL of 10X dCTP nucleotide mix, 3 µl of Cy3-dCTP (or 3 µl of Cy5-dCTP depending on the strain), 1 µl of exo-klenow fragment enzyme. The solution is mixed and quickly spinned down prior to incubation at 37°C for 2 hours. Reaction is stopped by addition of 5µl of stop buffer. Probes are purified through purification columns as recommended by the supplier of the kit. 45 µl of TE buffer are added as well as 400 µl of purification buffer A. Solution is vortexed for 30 sec and loaded on a purification column within a collection tube. The column is centrifuged at 11000 g for 1 min at room temperature and the flow-through is discarded. 600 µl of purification buffer B are added to the column prior to centrifugation at 11000 g for 1 min at room temperature and the flow-through is discarded. The purification column is placed in another collection tube and 50 µl of sterile distilled water is added. The column is incubated 1 min at room temperature and centrifuged at 11000 g for 1 min at room temperature. The flow-through contains the purified probe. *E. coli* DNA prepared and labelled in the very same way is used as control. When using Cy3-labelled DNA from *E. ruminantium* Gardel, the *E. coli* control DNA is labelled with Cy5-dCTP, whereas when using Cy5-labelled DNA from *E. ruminantium* Welgevonden, the *E. coli* control DNA is labelled with Cy3-dCTP.

Hybridization of DNA to the microarrays

The labelled DNA preparations, samples and control, (0.9-1.2 µg per strain) are denaturated at 95°C for 5 min. They are subsequently added to microarray hybridization buffer (Amersham Biosciences) and are applied to the microarrays in individual chambers of an automated slide processor (Amersham Biosciences). Hybridization is carried out at 42°C for PCR array (or 37 °C for oligos array) for 12 h. Hybridized slides are washed at 45°C successively with 1 X SSC, 0.2% SDS for 10 min, twice with 0.1 X SSC, 0.2% SDS for 10 min, with 0.1 X SSC for 1 min and with isopropanol before air drying. Microarrays are immediately scanned in both Cy3 and Cy5 channels with Amersham generation III array scanner with a 10 µm resolution.

Results

DNA from strain Gardel hybridizes with all the spots of the micro-array bearing the amplicons PCR-oligo-ERGA-4340, PCR-oligo-ERGA-4980, PCR-oligo-ERGA-5590, PCR-oligo-ERGA-5600, PCR-oligo-ERGA-7580 and does not hybridizes with the spots bearing the amplicon PCR-oligo-ERWE-8330. On the other hand, DNA from strain Welgevonden hybridizes only with the spot bearing the amplicon PCR-oligo-ERWE-8330

These results show that *E. ruminantium* strain Gardel can be specifically discriminated from *E. ruminantium* strain Welgevonden using any combination from 1 to 6 of these amplicons.

EXAMPLE 6. DIFFERENTIAL DNA ARRAY DETECTION OF STRAINS OF *E. RUMINANTIUM* BASED ON RECOGNITION OF ORPHAN GENES WITH OLIGONUCLEOTIDES

This example is based on the use of oligonucleotide probes specific to the orphan genes to be detected. Oligonucleotides were designed using OligoArray 2.1 (Rouillard *et al.*, Nucleic Acids Research. 31: 3057-3062, 2003). Table 6 below indicates the size of these oligonucleotides and their positions relative to the corresponding CDS.

TABLE 6

Oligonucleotide			CDS recognized by the oligonucleotide
Name	Size (bp)	Position relative to CDS sequence	
Oligo-ERGA-4340	50	137-186	ERGA_CDS_04340
Oligo-ERGA-4980	51	180-230	ERGA_CDS_04980
Oligo-ERGA-5590	50	561-610	ERGA_CDS_05590
Oligo-ERGA-5600	50	710-759	ERGA_CDS_05600
Oligo-ERGA-7580	50	194-243	ERGA_CDS_07580
Oligo-ERWE-8330	51	35-85	ERWE_CDS_08330

To prepare DNA arrays, the oligonucleotides are spotted on aminosilane-coated mirror glass slides (7 Star, Amersham Biosciences) following the procedure described for amplicons in Example 5 above. Labelled DNAs from *E. ruminantium* strain Gardel or *E. ruminantium* strain Welgevonden are prepared and hybridized to said arrays as disclosed in Example 5 above.

DNA from strain Gardel hybridizes with all the spots of the micro-array bearing the oligonucleotides Oligo-ERGA-4340, Oligo-ERGA-4980, Oligo-ERGA-5590, Oligo-ERGA-5600, Oligo-ERGA-7580 and does not hybridize with the spot bearing the oligonucleotide Oligo-ERWE-8330. On the other hand, DNA from strain Welgevonden hybridizes only with the spot bearing the oligonucleotide Oligo-ERWE-8330.

Thus *E. ruminantium* strain Gardel can be specifically discriminated from *E. ruminantium* strain Welgevonden using any combination from 1 to 6 of these oligonucleotides.

EXAMPLE 7. DIFFERENTIAL DNA ARRAY DETECTION OF STRAINS OF *E. RUMINANTIUM* BASED ON RECOGNITION OF MUTATED GENES WITH OLIGONUCLEOTIDES

The genes targeted in this example are truncated genes for which part of the transcribed region is lost in the central part of the initial coding sequence. Three main regions can therefore be considered. The first region, named Zone 1, is the 5' region of the gene up to the beginning of the deletion in the mutated gene. Zone 1 is a conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. An oligonucleotide designed to match this region (N1) recognizes both strains. Zone 2, corresponds to the region of deletion in the mutant allele and therefore only the native full length allele bears a sequence in this region and can be recognized by an oligonucleotide (N2). Zone 3 is the second conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. In this region also, an oligonucleotide designed to match Zone 3 (N3) recognizes both strains.

Oligonucleotides probes targeted to Zone 1, Zone 2, and Zone 3 of each of the allelic couples:

ERGA_CDS_01350/ERWE_CDS_01390,

ERGA_CDS_04500/(ERWE_CDS_04590 + ERWE_CDS_4600)

ERGA_CDS_05350/(ERWE_CDS_05460 + ERWE_CDS_5470)

were designed using OligoArray 2.1 (Rouillard *et al.*, cited above). Table 7 below indicates the size of these oligonucleotides and their positions relative to the corresponding CDS. The sequence of these oligonucleotides is indicated in Table 15A.

TABLE 7

Oligonucleotide name	Zone	Size (mer)	Position relative to CDS	Mutant allele	Position relative to CDS	Native allele
MutERWE-1390N1	1	50	507-556	ERWE_CDS_01390	507-556	ERGA_CDS_01350
MutERWE-1390N2	2	50	-	ERWE_CDS_01390	2328-2377	ERGA_CDS_01350
MutERWE-1390N3	3	50	2771-2820	ERWE_CDS_01390	3167-3216	ERGA_CDS_01350
MutERWE-4590N1	1	50	455-504	ERWE_CDS_04590	449-498	ERGA_CDS_04500
MutERWE-4590N2	2	50	-	ERWE_CDS_04590	536-585	ERGA_CDS_04500
MutERWE-4600N3	3	50	962-1011	ERWE_CDS_04600	2777-2826	ERGA_CDS_04500
MutERWE-5460N1	1	50	2251-2300	ERWE_CDS_05460	6793-6842	ERGA_CDS_05350
MutERWE-5460N2	2	50	-	ERWE_CDS_05460	5455-5504	ERGA_CDS_05350
MutERWE-5470N3	3	50	3440-3489	ERWE_CDS_05470	3440-3489	ERGA_CDS_05350

These oligonucleotides can be used as oligonucleotide multiplexes for DNA array detection of *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. These oligonucleotide multiplexes are listed in Table 8 below.

TABLE 8

Mut oligonucleotide mutiplexes	Oligonucleotides	Mutant allele	Native allele	Zone
MutERWE-1390	MutERWE 1390N1	ERWE_CDS_01390	ERGA_CDS_01350	1
	MutERWE 1390N2	ERWE_CDS_01390	ERGA_CDS_01350	2
	MutERWE 1390N3	ERWE_CDS_01390	ERGA_CDS_01350	3
MutERWE-4590/4600	MutERWE 4590N1	ERWE_CDS_04590	ERGA_CDS_04500	1
	MutERWE 4590N2	ERWE_CDS_04590	ERGA_CDS_04500	2
	MutERWE 4600N3	ERWE_CDS_04600	ERGA_CDS_04500	3
MutERWE-5460/5470	MutERWE 5460N1	ERWE_CDS_05460	ERGA_CDS_05350	1
	MutERWE 5460N2	ERWE_CDS_05460	ERGA_CDS_05350	2
	MutERWE 5470N3	ERWE_CDS_05470	ERGA_CDS_05350	3

To prepare DNA arrays, the oligonucleotides are spotted on aminosilane-coated mirror glass slides following the procedure described for amplicons in Example 5 above. Labelled DNAs from *E. ruminantium* strain Gardel or *E. ruminantium* strain Welgevonden are prepared and hybridized to said arrays as disclosed in Example 5 above.

A differential analysis on both strains is carried out by running a separate reaction for each oligonucleotide, N1, N2 and N3 in each strain making a total of six separate reactions, each on a specific spot (six separate spots). All members of the series hybridize with DNA from the strain bearing the native full length allele yielding three positive responses. The N1 and N3 oligonucleotides specific to the conserved Zone 1 and 3 hybridize with DNA from the strains bearing the mutation. The N2 oligonucleotide specific to the deleted region (Zone 2) does not hybridize onto the stain bearing the mutation and yields a negative response. A typical pattern for the strain bearing the mutation is a positive response for oligonucleotide N1 and N3 and a negative response for oligonucleotide N2.

The Mut oligonucleotide series described in this example allow differential discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden through DNA arrays on three different genes with three different series of three oligonucleotides. This provides a high level of confidence in the specific identification of each strain.

EXAMPLE 8. DIFFERENTIAL PCR DETECTION OF STRAINS OF *E. RUMINANTIUM*

This example illustrates the discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden based on the differential PCR amplification of native and mutant alleles of 6 different genes:

ERGA_CDS_00120/ERWE_CDS_00120

ERGA_CDS_01350/ERWE_CDS_01390

ERGA_CDS_05740/ERWE_CDS_05830

ERGA_CDS_04500/(ERWE_CDS_04590 + ERWE_CDS_4600)

ERGA_CDS_05350/(ERWE_CDS_05460 + ERWE_CDS_5470)

ERGA_CDS_07330/ERWE_CDS_07410

Primers were designed using VectorNTI Advance 9.0 (Informax).

Table 9 below provides a list of these primers and indicates their positions relative to the corresponding CDS. The sequence of these PCR primers is indicated in Table 15B.

TABLE 9.

Primer (orientation)	Zone	Position relative to CDS	Native allele	Position relative to CDS	Mutant allele
P-Z-1-ERGA-120 (sense)	1	5-29	ERGA_CDS_00120	5-29	ERWE_CDS_00120
P-Z-2-ERGA-120-S (sense)	2	442-466	ERGA_CDS_00120	-	ERWE_CDS_00120
P-Z-2-ERGA-120-AS (antisense)	2	442-466	ERGA_CDS_00120	-	ERWE_CDS_00120
P-Z-3-ERGA-120 (antisense)	3	1222-1246	ERGA_CDS_00120	1132-1156	ERWE_CDS_00120
P-Z-1-ERGA-1350 (sense)	1	240-264	ERGA_CDS_01350	240-264	ERWE_CDS_01390
P-Z-2-ERGA-1350-S (sense)	2	2344-2368	ERGA_CDS_01350	-	ERWE_CDS_01390
P-Z-2-ERGA-1350-AS (antisense)	2	2344-2368	ERGA_CDS_01350	-	ERWE_CDS_01390
P-Z-3-ERGA-1350 (antisense)	3	3007-3031	ERGA_CDS_01350	2611-2635	ERWE_CDS_01390
P-Z-1-ERGA-4500 (sense)	1	301-325	ERGA_CDS_04500	307-331	ERWE_CDS_04590
P-Z-2-ERGA-4500-S (sense)	2	548-572	ERGA_CDS_04500	-	ERWE_CDS_04590
P-Z-2-ERGA-4500-AS (antisense)	2	548-572	ERGA_CDS_04500	-	ERWE_CDS_04590
P-Z-3-ERGA-4500 (antisense)	3	829-853	ERGA_CDS_04500	775-799	ERWE_CDS_04590
P-Z-1-ERGA-5350 (sense)	1	4525-4549	ERGA_CDS_05350	223-247	ERWE_CDS_05460
P-Z-2-ERGA-5350-S (sense)	2	5466-5491	ERGA_CDS_05350	-	ERWE_CDS_05460
P-Z-2-ERGA-5350-AS (antisense)	2	5466-5491	ERGA_CDS_05350	-	ERWE_CDS_05460
P-Z-3-ERGA-5350 (antisense)	3	6071-6095	ERGA_CDS_05350	1532-1556	ERWE_CDS_05460
P-Z-1-ERGA-5740 (sense)	1	443-467	ERGA_CDS_05740	449-473	ERWE_CDS_05830
P-Z-2-ERGA-5740-S (sense)	2	1376-1400	ERGA_CDS_05740	-	ERWE_CDS_05830
P-Z-2-ERGA-5740-AS (antisense)	2	1376-1400	ERGA_CDS_05740	-	ERWE_CDS_05830
P-Z-3-ERGA-5740 (antisense)	3	1780-1804	ERGA_CDS_05740	1603-1627	ERWE_CDS_05830
P-Z-1-ERWE-7410 (sense)	1	151-175	ERWE_CDS_07410	151-175	ERGA_CDS_07330
P-Z-2- ERWE-7410-S (sense)	2	639-663	ERWE_CDS_07410	-	ERGA_CDS_07330
P-Z-2- ERWE-7410-AS (antisense)	2	639-663	ERWE_CDS_07410	-	ERGA_CDS_07330
P-Z-3- ERWE-120 (antisense)	3	1818-1843	ERWE_CDS_07410	1222-1246	ERGA_CDS_07330

P-Z-1 and P-Z-3 primers recognize the conserved regions Zone 1 and Zone 3, respectively, in both *E. ruminantium* strain Gardel and *E. ruminantium* strain

Welgevonden. P-Z-2 primers are specifically binding to Zone 2 in the native full length allele and do not hybridise to the genome of the strain bearing the corresponding mutant allele.

In this example, P-Z-1 primers are sense primers whereas P-Z-3 primers are antisense primers. Two kinds of P-Z-2 primers are used, sense primer (labelled –S) and antisense primers (labelled –AS). They recognized the same region but on complementary strands and directed amplification in opposite orientations.

Two different ways of use of these primers are exemplified herein: simple PCR and multiplex PCR.

Simple PCR

DNA is extracted from *E. ruminantium* elementary bodies, as described in example 5 above. Simple direct PCR is performed using a standard procedure (Sambrook & Russel, Molecular Cloning "a laboratory manual", 3rd Edition, vol. 2, Chapter 8).

250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense and antisense, primer and 3 mM $MgCl_2$ are mixed in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

The amplification patterns obtained using three different pairs of primers (P-Z-1 + P-Z-3, P-Z-1 + P-Z-2-AS and P-Z-3 + P-Z-2-S) are shown in Table 10.

TABLE 10.

Primer pairs	E. ruminantium strain Gardel (size)	E. ruminantium strain Welgevonden (size)
P-Z-1-ERGA-120 + P-Z-3-ERGA-120	Positive (1242 bp)	Positive (1152 bp)
P-Z-1-ERGA-120 + P-Z-2-ERGA-120-AS	Positive (462 bp)	Negative
P-Z-3-ERGA-120 + p-Z-2-ERGA-120-S	Positive (805 bp)	Negative
P-Z-1-ERGA-1350 + P-Z-3-ERGA-1350	Positive (2792 bp)	Positive (2396 bp)
P-Z-1-ERGA-1350 + P-Z-2-ERGA-1350-AS	Positive (2129 bp)	Negative
P-Z-3-ERGA-1350 + P-Z-2-ERGA-1350-S	Positive (688 bp)	Negative
P-Z-1-ERGA-4500 + P-Z-3-ERGA-4500	Positive (553 bp)	Positive (493 bp)
P-Z-1-ERGA-4500 + P-Z-2-ERGA-4500-AS	Positive (272 bp)	Negative
P-Z-3-ERGA-4500 + P-Z-2-ERGA-4500-S	Positive (306 bp)	Negative
P-Z-1-ERGA-5350 + P-Z-3-ERGA-5350	Positive (1571 bp)	Positive (1334bp)
P-Z-1-ERGA-5350 + P-Z-2-ERGA-5350-AS	Positive (967 bp)	Negative
P-Z-3-ERGA-5350 + P-Z-2-ERGA-5350-AS	Positive (630 bp)	Negative
P-Z-1-ERGA-5740 + P-Z-3-ERGA-5740	Positive (1362 bp)	Positive (1179 bp)
P-Z-1-ERGA-5740 + P-Z-2-ERGA-5740-AS	Positive (958 bp)	Negative
P-Z-3-ERGA-5740 + P-Z-2-ERGA-5740-S	Positive (429 bp)	Negative
P-Z-1-ERWE-7410 + P-Z-3-ERWE-7410	Positive (1096 bp)	Positive (1693 bp)
P-Z-1-ERWE-7410 + P-Z-2-ERWE-7410-AS	Negative	Positive(513 bp)
P-Z-3-ERWE-7410 + P-Z-2-ERWE-7410-S	Negative	Positive(1205 bp)

For each doublet of native and mutant alleles, *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden can be discriminated according to the expected size of the amplification products.

Multiplex PCR:

Differential detection through multiplex PCR is performed on DNA prepared from *E. ruminantium* elementary bodies as described in Example 5 above.

For each couple of allele, the multiplex PCR is carried out by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of P-Z-1 primer, 0.5 µM of P-Z-2 primer, 0.5 µM of P-Z-3 primer and 3 mM $MgCl_2$ in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products, i.e. doublets, are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

The use, for any of the targeted gene, of a P-Z-1 sense primer and two antisense primers, i.e. P-Z-2-AS and P-Z-3, generates two PCR products of differing size in the same reaction when the gene present in the strain is the native full length allele. These two PCR products correspond to an amplification driven by the P-Z-1/P-Z-2-AS pair and by the P-Z-1/P-Z-3 pair. When a mutant allele is present, the PCR reaction generates only one amplification product driven by the P-Z-1/P-Z-3 pair.

The amplification patterns obtained on *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden are shown in Table 11.

TABLE 11.

Primer triplets	E. ruminantium strain Gardel (size)	E. ruminantium strain Welgevonden (size)
P-Z-1-ERGA-120 + P-Z-2-ERGA-120-AS + P-Z-3-ERGA-120	2 PCR products (1242 bp and 462 bp)	1 PCR product (1172 bp)
P-Z-1-ERGA-1350 + P-Z-2-ERGA-1350-AS + P-Z-3-ERGA-1350	2 PCR products (2792 bp and 2129 bp)	1 PCR product (2396 bp)
P-Z-1-ERGA-4500 + P-Z-2-ERGA-4500-AS + P-Z-3-ERGA-4500	2 PCR products (553 bp and 272 bp)	1 PCR product (493 bp)
P-Z-1-ERGA-5350 + P-Z-2-ERGA-5350-AS + P-Z-3-ERGA-5350	2 PCR products (1571 bp and 967 bp)	1 PCR product (1334 bp)
P-Z-1-ERGA-5740 + P-Z-2-ERGA-5740-AS + P-Z-3-ERGA-5740	2 PCR products (1362 bp and 958 bp)	1 PCR product (1179 bp)
P-Z-1-ERWE-7410 + P-Z-2-ERWE-7410-AS + P-Z-3-ERWE-7410	1 PCR product (1096 bp)	2 PCR products (1693 bp and 513 bp)

In the same way as for the simple PCR described above, *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden can be discriminated according to the size of the amplification products.

EXAMPLE 9. DIFFERENTIAL DISCRIMINATION BETWEEN *E. RUMINANTIUM* STRAIN GARDEL AND *E. RUMINANTIUM* STRAIN WELGEVONDEN BASED ON RFLP AND SEQUENCE ANALYSIS-RELATED MEANS

This example illustrates the discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden by PCR amplification of the members of same couples of genes as in example 8, followed by RFLP analysis of the amplification products.

Primers targeted to sequences that are present in both *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden and giving amplification products of different sizes depending on the strain, were designed using VectorNTI Advance 9.0 (Informax).

Table 12 below provides a list of these primers and indicates their positions relative to the corresponding CDS. The sequence of these PCR primers is indicated in Table 15B.

TABLE 12.

Primers	Orientation	Position relative to CDS Strain Gardel	Position relative to CDS Strain Welgevonden
P-WEGA-120-S	Sense	1-25 (ERGA_CDS_00120)	1-25 (ERWE_CDS_00120)
P-WEGA-120-AS	Antisense	1237-1261(ERGA_CDS_00120)	1147-1171 (ERWE_CDS_00120)
P-WEGA-1350-S	Sense	1430-1454 (ERGA_CDS_01350)	1430-1454 (ERWE_CDS_01390)
P-WEGA-1350-AS	Antisense	2887-2911(ERGA_CDS_01350)	2491-2515 (ERWE_CDS_01390)
P-WEGA-4500-S	Sense	301-325 (ERGA_CDS_04500)	307-331 (ERWE_CDS_04590)
P-WEGA-4500-AS	Antisense	3541-3565 (ERGA_CDS_04500)	1711-1735 (ERWE_CDS_04600)
P-WEGA-5350-S	Sense	3365-3389 (ERGA_CDS_05350)	3365-3389 (ERWE_CDS_05470)
P-WEGA-5350-AS	Antisense	6079-6103 (ERGA_CDS_05350)	1540-1564 (ERWE_CDS_05460)
P-WEGA-5740-S	Sense	241-268 (ERGA_CDS_05740)	244-271 (ERWE_CDS_05830)
P-WEGA-5740-AS	Antisense	1779-1806 (ERGA_CDS_05740)	1602-1629 (ERWE_CDS_05830)
P-WEGA-7410-S	Sense	1-25 (ERGA_CDS_07330)	1-25 (ERWE_CDS_07410)
P-WEGA-7410-AS	Antisense	1365-1389 (ERGA_CDS_07330)	1962-1986 (ERWE_CDS_07410)

PCR amplification

PCR amplification is carried out, for each couple of allele, by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense (S) and antisense (AS) primer, and 3 mM $MgCl_2$ in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products are checked by running an aliquot on 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

The P-WEGA-120-S/P-WEGA-120-AS pair directs the amplification of a 1261 bp PCR product from ERGA_CDS_00120 in *E. ruminantium* strain Gardel (i.e. RFLP-ERGA-120) and a 1171 bp PCR product from ERWE_CDS_00120 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-120).

The P-WEGA-1350-S/P-WEGA-1350-AS pair yields a 1482 bp PCR product from ERGA_CDS_01350 in *E. ruminantium* strain Gardel (i.e. RFLP-ERGA-1350)

and a 1086 bp PCR product from ERWE_CDS_1390 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-1390).

The P-WEGA-4500-S/P-WEGA-4500-AS pair drives amplification of a 3265 bp PCR product from ERGA_CDS-04500 in *E. ruminantium* strain Gardel (RFLP-ERGA-4500) and a 2675 bp PCR product from ERWE_CDS-04590/ERWE_CDS_04600 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-4590/4600).

The P-WEGA-5350-S/P-WEGA-5350-AS pair drives amplification of a 2739 bp PCR product from ERGA_CDS-05350 in *E. ruminantium* strain Gardel (RFLP-ERGA-05350) and a 2765 bp PCR product from ERWE_CDS-05460/ERWE_CDS_05470 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-5460/5470).

The P-WEGA-5740-S/P-WEGA-5740-AS pair drives amplification of a 1566 bp PCR product from ERGA_CDS-05740 in *E. ruminantium* strain Gardel (RFLP-ERGA-05740) and a 1386 bp PCR product from ERWE_CDS-05830 in *E. ruminantium* strain Welgevonden (RFLP-ERWE-5830).

Similarly, P-WEGA-7410-S/P-WEGA-7410-AS pair drives amplification of a 1389 bp PCR product from ERGA_CDS-07330 (RFLP-ERGA-07330), and a 1986 bp PCR product from ERWE_CDS-07410 in *E. ruminantium* strain Welgevonden (RFLP-ERWE-7410).

These PCR products are listed in Table 13 below.

TABLE 13

PCR-product	Sense-primer	Antisense-primer	Gene-detected	Strain
RFLP-ERGA-120	P-WEGA-120-S	P-WEGA-120-AS	ERGA_CDS_00120	Gardel
RFLP-ERWE-120	P-WEGA-120-S	P-WEGA-120-AS	ERWE_CDS_00120	Welgevonden
RFLP-ERGA-1350	P-WEGA-1350-S	P-WEGA-1350-AS	ERGA-CDS_01350	Gardel
RFLP-ERWE-1390	P-WEGA-1350-S	P-WEGA-1350-AS	ERWE_CDS_01390	Welgevonden
RFLP-ERGA-4500	P-WEGA-4500-S	P-WEGA-4500-AS	ERGA_CDS_04500	Gardel
RFLP-ERWE-4590/4600	P-WEGA-4500-S	P-WEGA-4500-AS	ERWE_CDS_04590 ERWE_CDS_04600	Welgevonden
RFLP-ERGA-5350	P-WEGA-5350-S	P-WEGA-5350-AS	ERGA_CDS_05350	Gardel
RFLP-ERWE-5460/5470	P-WEGA-5350-S	P-WEGA-5350-AS	ERWE_CDS_05460 ERWE_CDS_05470	Welgevonden
RFLP-ERGA-5740	P-WEGA-5740-S	P-WEGA-5740-AS	ERGA_CDS_05740	Gardel
RFLP-ERWE-5830	P-WEGA-5740-S	P-WEGA-5740-AS	ERWE_CDS_05830	Welgevonden
RFLP-ERGA-7330	P-WEGA-7410-S	P-WEGA-7410-AS	ERGA_CDS_07330	Gardel
RFLP-ERWE-7410	P-WEGA-7410-S	P-WEGA-7410-AS	ERWE_CDS_07410	Welgevonden

RFLP analysis

The PCR products are used for further RFLP analysis with the following restriction endonucleases: *Alu*I, *Dra*I, *Eco*RV, *Hin*fI, *Rsa*I and *Taq*I.

PCR products are digested in a final volume of 20 µl with the selected enzyme under the conditions, i.e. buffer, time and temperature, recommended by the supplier of the enzyme. Following digestion, the restriction fragments are separated on 2% agarose gel electrophoresis in Tris-borate-EDTA buffer.

The results are shown in Table 14 below.

TABLE 14

PCR product	Number of sites for selected restriction enzymes					
	AluI	DraI	EcoRV	HinfI	RsaI	TaqI
RFLP-ERGA-120	4	2	4	11	8	4
RFLP-ERWE-120	4	2	1	9	9	1
RFLP-ERGA-1350	8	2	1	10	14	5
RFLP-ERWE-1390	5	4	1	1	10	1
RFLP-ERGA-4500	6	5	4	10	14	5
RFLP-ERWE-4590/4600	5	9	1	8	8	6
RFLP-ERGA-5350	5	2	2	6	13	2
RFLP-ERWE-5460/5470	2	2	2	8	17	2
RFLP-ERGA-5740	2	4	1	8	17	2
RFLP-ERWE-5830	3	3	2	6	12	4
RFLP-ERGA-7330	2	3	1	2	9	1
RFLP-ERWE-7410	2	3	1	2	17	1

These results show that depending on the strain's DNA used as template for the PCR, all the PCR products yield a different number of bands for at least one of the tested restriction enzymes.

EXAMPLE 10. DISCRIMINATION BETWEEN *E. RUMINANTIUM* STRAIN GARDEL AND *E. RUMINANTIUM* STRAIN WELGEVONDEN BASED ON DNA HYBRIDIZATION

This example illustrates the use of the Mut oligonucleotide series described in Example 7 as labelled DNA probes to specifically discriminate *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden through DNA hybridization analysis.

The Mut oligonucleotides used as DNA probes are labelled with α-32P dCTP by random priming using the Rediprime II DNA labeling system (Amersham Bioscience) following the procedure described by the supplier. DNA concentration is adjusted so that 45 µl of DNA solution in TE buffer (10 mM Tris-HCl, pH 8.0) contains 25 ng of DNA. DNA is denatured by incubation at 100°C for 5 min, followed by immediate incubation in ice-cold water for 5 min. The tube is briefly centrifuged to bring the contents to the bottom of the tube. Denatured DNA is transferred to the reaction tube provided in the kit and mixed to 5 µl of Redivue [32P] dCTP. Reaction is incubated at 37°C for 10 min and stopped by addition of 5 µl of 0.2 m EDTA. The labelled probe is heat denatured by incubation at 100°C for 5 min followed by incubation for 5 min in ice-cold water prior to hybridization with *E. ruminantium* DNA.

Total DNA from strain Gardel or from strain Welgevonden is obtained as described in Example 5.

Heat denatured DNA is spotted on a nylon membrane (Hybond N+, Amersham) using a slot blot manifold (Hoeffer Scientific Instruments) following the procedure recommended by the supplier of the membrane (Amersham). DNA-DNA hybridization is conducted as described in the Hybond N+ user manual. Prehybridization is conducted overnight in an hybridization tube (meant for use in hybridization ovens) and incubated at 68°C with 20 ml of prehybridization buffer (Denhart 5x, SSPE 2x, SDS .5x,

SSPE-Dextran 4x, denatured hering-sperm DNA). After removal of the prehybridization buffer, the membrane is incubated overnight at 68°C in 10 ml of hybridization buffer (Denhart 5x, SSPE 2x, SDS .5x, SSPE-Dextran 4x, denatured hering-sperm DNA, 30 ng/ml of labelled DNA probe). Following hybridization, the membrane is washed successively for 30 min in 2x SSC, 0.5 % SDS at room temperature and in 0.1x SSC, 0.5 % SDS at 68°C.

Hybridized probes are revealed by autoradiography.

MutERWE 1390N1, MutERWE 1390N3, MutERWE 4590N1, MutERWE 4600N3, MutERWE 5460N1, and MutERWE 5470N3, hybridize with DNA from both strains Gardel and Welgevonden, while MutERWE 1390N2, MutERWE 4590N2, MutERWE 5460N2 only hybridize with DNA from strain Gardel.

TABLE 15A

Oligonucleotide or Primer name	Orientation	Sequence (from 5' to 3')	SEQ ID NO
P-ERGA-4340-A	sense	atgagtcacagtttatttgag	21
P-ERGA-4340-B	antisense	cactcaaaatcacaaggaagta	22
P-ERGA-4980-A	sense	atgtatttagtctatttagtagctg	23
P-ERGA-4980-B	antisense	ataacatctaattgaacaatatc	24
P-ERGA-5590-A	sense	atgaaaggatctttatctgc	25
P-ERGA-5590-B	antisense	ccttcttcttcttcattatg	26
P-ERGA-5600-A	sense	aagaattacatgatgcagc	27
P-ERGA-5600-B	antisense	tcttctcttgttatactctctg	28
P-ERGA-7580-A	sense	atggatttaaataaactaataaa	29
P-ERGA-7580-B	antisense	gcattttctctacctacga	30
P-ERWE-8330-A	sense	gtctttatataaaagtaagaattga	31
P-ERWE-8330-B	antisense	tgctataagattgaactgaaa	32
Oligo-ERGA-4340	sense	cactaattaacaatattacttcttgtgattttgagtgtaataaacaatga	33
Oligo-ERGA-4980	sense	gttaaatttaatgtcagatattgttcaattagatgttataatgttaaaagg	34
Oligo-ERGA-5590	sense	aggtcgtggtcttgctttttccatgatgttgcaagtaattttgaaacat	35
Oligo-ERGA-5600	sense	gtaaacaagaggaaggattagaaacacatcagctttccaccaatgtagta	36
Oligo-ERGA-7580	sense	ttgaggattttatgttctcagaacaaatcgtaggtagagaaaatgcagaa	37
Oligo-ERWE-8330	sense	ttgatgattctactgatgttattacttataactctaaaaaaaaatatgtgta	38
MutERWE-1390N1	sense	tgatgttacagatagattgtatgtgatgtggcaattgagatatcataata	39
MutERWE-1390N2	sense	tgtaataaagcctactcactatgtaacgcatgtaacattggaatcgaagt	40
MutERWE-1390N3	sense	tttttaatttggatagtattcaaagtagtgtttctggtgtgcaagtgaca	41
MutERWE-4590N1	sense	ttcctattaacatagaacatgctctatcaaatatagcaaatttaaatgca	42
MutERWE-4590N2	sense	atctaataaatgcgtctgatctaataaatgcgtctgatctaataaaagaa	43
MutERWE-4600N3	sense	tcatcaaaaagatacgttgtataggtaatactatagatcctgaacaagga	44
MutERWE-5460N1	sense	tctttaaaagataaaaaatcaaagcttactgatcctagtgagatagcaaa	45
MutERWE-5460N2	sense	gaacaagataaggtaggagaatttgaagtagctgaagatactagtgtaga	46
MutERWE-5470N3	sense	gtgcttctgttccagatacaggacaagatatattacatagtaatgctgct	47

TABLE 15B

Oligonucleotide or Primer name	Orientation	Sequence (from 5' to 3')	SEQ ID NO
P-Z-1-ERGA-120	sense	gtattgataattatgatggtgaaac	48
P-Z-2-ERGA-120-S	sense	gcacatgatatcgaacatgcagttc	49
P-Z-2-ERGA-120-AS	antisense	gaactgcatgttcgatatcatgtgc	50
P-Z-3-ERGA-120	antisense	ggttacaaggacaatgatgagtgtg	51
P-Z-1-ERGA-1350	sense	tccaccagagatgttatttgtaaag	52
P-Z-2-ERGA-1350-S	sense	cactatgtaacgcatgtaacattgg	53
P-Z-2-ERGA-1350-AS	antisense	ccaatgttacatgcgttacatagtg	54
P-Z-3-ERGA-1350	antisense	caacagaactttcagtattaaaagc	55
P-Z-1-ERGA-4500	sense	gttaagtgtgaaatgtattgtttag	56
P-Z-2-ERGA-4500-S	sense	cgtctgatctaataaatgcgtctga	57
P-Z-2-ERGA-4500-AS	antisense	tcagacgcatttattagatcagacg	58
P-Z-1/3-ERGA-4500-S	sense	ctagtaaggaaagaaaaacttaagc	59
P-Z-1/3-ERGA-4500-AS	antisense	gcttaagtttttctttccttactag	60
P-Z-3-ERGA-4500	antisense	cactttctgttaattcaaaagtaga	61
P-Z-1-ERGA-5350	sense	gaattaattgatatgaatgcagaag	62
P-Z-2-ERGA-5350-S	sense	ggtaggagaatttgaagtagctgaag	63
P-Z-2-ERGA-5350-AS	antisense	cttcagctacttcaaattctcctacc	64
P-Z-3-ERGA-5350	antisense	cttgtagattcttcttctgtgctac	65
P-Z-1-ERGA-5740	sense	gtaggccaaaaagtataggtaatag	66
P-Z-2-ERGA-5740-S	sense	ttagaccaaaaacatttgcatctag	67
P-Z-2-ERGA-5740-AS	antisense	ctagatgcaaatgtttttggtctaa	68
P-Z-3-ERGA-5740	antisense	caacaaatacatcatcttcaagttg	69
P-Z-1-ERWE-7410	sense	agggttacttattgtagtcagagtg	70
P-Z-2-ERWE-7410-S	sense	gagaagggatgttactgatacagcg	71
P-Z-2-ERWE-7410-AS	antisense	cgctgtatcagtaacatcccttctc	72
P-Z-3-ERWE-7410	antisense	cctcttcgtatacaggattaccatt	73
P-WEGA-120-S	sense	atgggtattgataattatgatggtg	74
P-WEGA-120-AS	antisense	caaatgtaatttcatggttacaagg	75
P-WEGA-1350-S	sense	gcgatgttataactgtttcaggtaa	76
P-WEGA-1350-AS	antisense	catgagatgtatatcttgtactcac	77
P-WEGA-4500-S	sense	gttaagtgtgaaatgtattgtttag	78
P-WEGA-4500-AS	antisense	ctaaatctttactttgagatttatg	79
P-WEGA-5350-S	sense	atttatcagcgactgattattctag	80
P-WEGA-5350-AS	antisense	ctagtacacttgtagattcttcttc	81
P-WEGA-5740-S	sense	cgtaatatatctttacaaaagttgacac	82
P-WEGA-5740-AS	antisense	ttcaacaaatacatcatcttcaagttga	83
P-WEGA-7410-S	sense	atgaatgagataatcctatacacag	84
P-WEGA-7410-AS	antisense	agtcacatcatattgactatgcaca	85

CLAIMS

1) A method for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden, wherein said method comprises the detection of the presence or the absence, in the bacteria to be tested, of at least one orphan gene selected among:

ERGA_CDS_04340 (SEQ ID NO: 1)

ERGA_CDS_04980 (SEQ ID NO: 2)

ERGA_CDS_05590 (SEQ ID NO: 3)

ERGA_CDS_05600 (SEQ ID NO: 4)

ERGA_CDS_07580 (SEQ ID NO: 5)

ERWE_CDS_08330 (SEQ ID NO: 6)

2) The method of claim 1, which further comprises the detection in the bacteria to be tested, of one of the members of at least one allelic couple of genes selected among :

- a couple consisting of ERGA_CDS_00120 (SEQ ID NO: 7) and ERWE_CDS_00120 (SEQ ID NO: 8);

- a couple consisting of ERGA_CDS_01350 (SEQ ID NO: 9) and ERWE_CDS_01390 (SEQ ID NO: 10);

- a couple consisting of ERGA_CDS_05740 (SEQ ID NO: 11) and ERWE_CDS_05830 (SEQ ID NO: 12);

- a couple consisting of ERGA_CDS_04500 (SEQ ID NO: 13) and ERWE_CDS_04590 (SEQ ID NO: 14) + ERWE_CDS_04600 (SEQ ID NO: 15)

- a couple consisting of ERGA_CDS_05350 (SEQ ID NO: 16) and ERWE_CDS_05460 (SEQ ID NO: 17) + ERWE_CDS_05470 (SEQ ID NO: 18)

- a couple consisting of ERGA_CDS_07330 (SEQ ID NO: 19) and ERWE_CDS_07410 (SEQ ID NO: 20).

3) An isolated polynucleotide selected among:

a) ERGA_CDS_05590, ERGA_CDS_07580, ERGA_CDS_04980, ERGA_CDS_05600, ERGA_CDS_08330, ERGA_CDS_04340, or their complement;

b) a fragment of at least 15 consecutive base pairs of a polynucleotide a);

c) a polynucleotide of at least 15 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a).

4) A DNA array comprising at least one polynucleotide selected among:

a) ERGA_CDS_05590, ERGA_CDS_07580, ERGA_CDS_04980, ERGA_CDS_05600, ERGA_CDS_08330, and ERGA_CDS_04340, or their complement;

b) a fragment of at least 30 consecutive base pairs of a polynucleotide a);

c) a polynucleotide of at least 30 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a).

5) A DNA array of claim 4 further comprising at least one polynucleotide selected among:

a) the portion of ERGA_CDS_01350, ERGA_CDS_04500, or ERGA_CDS_05350 that is deleted in *E. ruminantium* strain Welgevonden;

b) a fragment of at least 30 consecutive base pairs of a polynuclotide a);

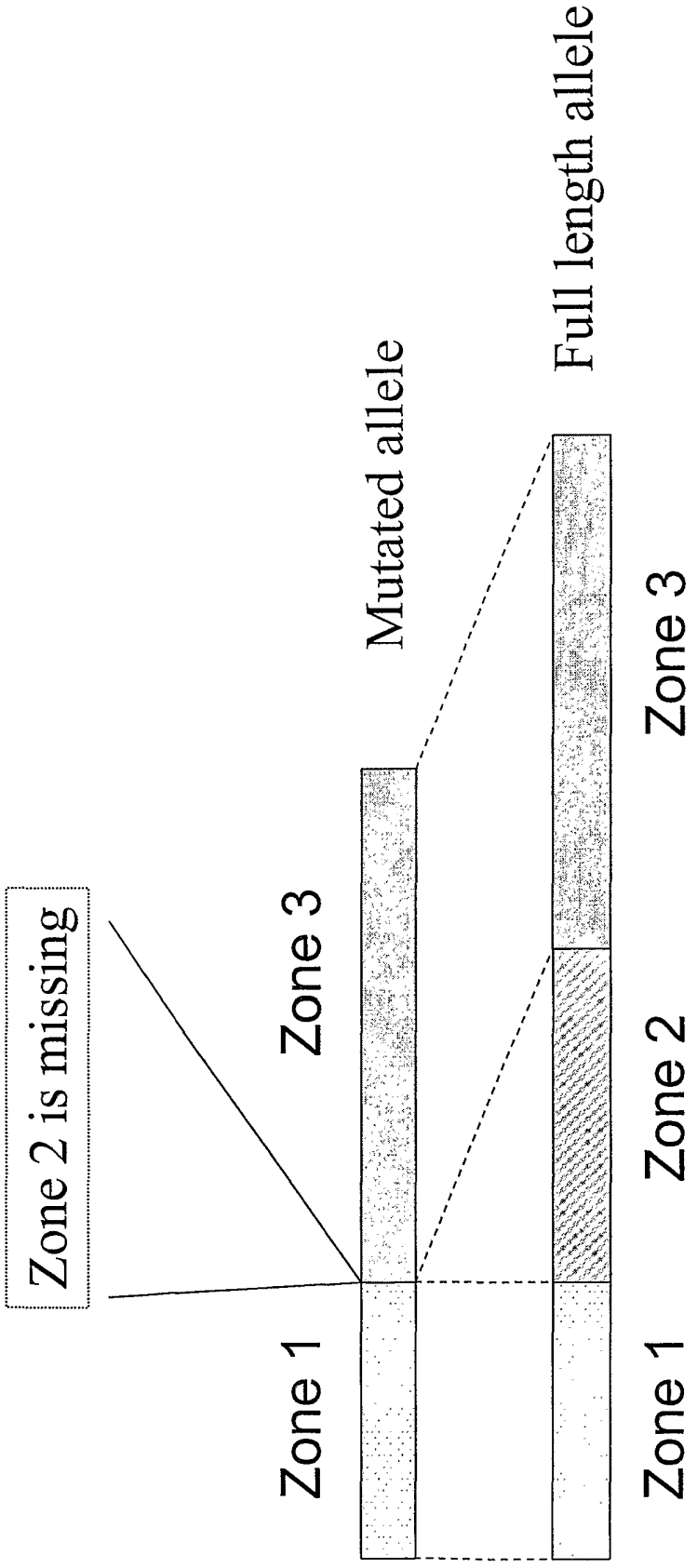
c) a polynucleotide of at least 30 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a).

6) A DNA array of claim 5, further comprising at least one polynucleotide selected among:

a) a portion of ERGA_CDS_01350, ERGA_CDS_04500, or ERGA_CDS_05350 that is conserved between *E. ruminantium* strains Gardel and Welgevonden;

b) a fragment of at least 30 consecutive base pairs of a polynuclotide a);

c) a polynucleotide of at least 30 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a).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Mutated allele
Full length allele
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Figure 1

SEQUENCE LISTING

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Centre National de la Recherche Scientifique (CNRS)
FRUTOS, Roger
FERRAZ, Conception
DEMAILLE, Jacques
MARTINEZ, Dominique

<120> SEQUENCES FOR DIFFERENTIAL DIAGNOSTIC OF EHRLICHIA RUMINANTIUM AND USE THEREOF

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<211> 3252
<212> DNA
<213> Ehrlichia ruminantium

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<211> 2856
<212> DNA
<213> Ehrlichia ruminantium

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tctatttatt atatgaaaat taatgatcat gcaattattg atcgtcggac aacaaattat 240
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gaagaaagta aaggtcgtaa agctttagtt atcaaaatat ataagataaa ttataatcct 360

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ttgagatatc ataataatag atttgaaatt ataggtttaa gtaatgggta taggcaattt 600
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<211> 1836
<212> DNA
<213> Ehrlichia ruminantium

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<210> 12
<211> 1659
<212> DNA
<213> Ehrlichia ruminantium

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<210> 13
<211> 3570
<212> DNA

<213> Ehrlichia ruminantium

<400> 13

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<400> 85
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/013853

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data, PAJ, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ALLSOPP M T ET AL: "Ehrlichia ruminantium major antigenic protein gene (map1) variants are not geographically constrained and show no evidence of having evolved under positive selection pressure." JOURNAL OF CLINICAL MICROBIOLOGY. NOV 2001, vol. 39, no. 11, November 2001 (2001-11), pages 4200-4203, XP002321870 ISSN: 0095-1137 page 4200, left-hand column, paragraph 1; figure 1; table 1	1-6

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2005	06/04/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Seroz, T

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BARBET A F ET AL: "A subset of Cowdria ruminantium genes important for immune recognition and protection" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER SCIENCE PUBLISHERS, BARKING, GB, vol. 275, no. 2, 19 September 2001 (2001-09-19), pages 287-298, XP004307852 ISSN: 0378-1119	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/013853

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-6 (all partially)

   An isolated polynucleotide as set forth in SEQ ID No 1 (ERGA_CDS_04340) and uses thereof.

   ---

2. claims: 1-6 (all partially)

   An isolated polynucleotide as set forth in SEQ ID No 2 (ERGA_CDS_04980) and uses thereof.

   ---

3. claims: 1-6 (all partially)

   An isolated polynucleotide as set forth in SEQ ID No 3 (ERGA_CDS_05590) and uses thereof.

   ---

4. claims: 1-6 (all partially)

   An isolated polynucleotide as set forth in SEQ ID No 4 (ERGA_CDS_05600) and uses thereof.

   ---

5. claims: 1-6 (all partially)

   An isolated polynucleotide as set forth in SEQ ID No 5 (ERGA_CDS_07580) and uses thereof.

   ---

6. claims: 1-6 (all partially)

   An isolated polynucleotide as set forth in SEQ ID No 6 (ERWE_CDS_08330) and uses thereof.
